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TITLE: Real-Time PCR Analysis of Peripheral Blood: A  
Non-Invasive Technology That Can Be Used to Assess Tumor  
Burden in Breast Cancer Patients

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<b>13. ABSTRACT (Maximum 200 Words)</b> <b>INTRODUCTION:</b> The molecular detection of circulating tumor cells (CTC) in breast cancer patients has been limited by two factors: 1) low concentration of CTC in peripheral blood, and 2) normal lymphocytes express some cancer-associated genes at detectable levels. In this study, we investigated the ability of a novel bouyant density gradient centrifugation (BDGC) medium (Oncoquick, HEXAL Gentech) to enrich CTC prior to real-time RT-PCR analysis. <b>METHODS:</b> MDA361 breast cancer cells were spiked into normal PBL by micromanipulation, and processed using Ficoll or BDGC medium. Tumor cell enrichment was assessed with flow cytometry, standard cytology, and real-time RT-PCR. <b>RESULTS:</b> BDGC medium dramatically reduced the number of recovered mononuclear cells. In a pilot study, 64% of Stage IV breast cancer patients and 100% of those patients naïve to chemotherapy had evidence of overexpression of at least one of five cancer-associated genes in peripheral blood. <b>CONCLUSIONS:</b> The use of bouyant density gradient centrifugation media: 1) results in superior reduction in background expression of cancer-associated gene from lymphocytes, 2) enhances tumor cell detection beyond established limits, and 3) has been successfully applied in Stage IV disease. Given the overall efficacy of Oncoquick, its use in Stage I-II disease is promising.				
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## INTRODUCTION

Recent advances in molecular genetics indicate that sporadic breast cancer can be characterized by the overexpression of certain genes or 'markers'. For example, using immunohistochemistry or reverse transcription polymerase chain reaction (RT-PCR) techniques, breast cancer cells have been shown to overexpress mammaglobin, CEA, VEGF, CK19, or Erb2B. Furthermore, pilot studies suggest that detection of these markers in the axillary lymph nodes (ALNs) or bone marrow of breast cancer patients correlates with poor outcome. These findings provide impetus for the development of screening assays to quantify expression levels of breast cancer-associated genes in the peripheral blood, arguably the most accessible tissue compartment for cancer. Sensitive detection and quantification of circulating breast cancer cells in the peripheral blood could be used 1) for the early detection of breast cancer, 2) to monitor the response to treatment, and 3) for the detection of recurrent disease. Thus, the successful development and validation of a screening assay would have a significant clinical impact.

## BODY

**Aim 1: Develop technology for real-time PCR detection of breast tumor cells in peripheral blood.**

### Two compartment separation results in dramatic enrichment of breast cancer cells from peripheral blood.

To assess the efficacy of tumor cell detection in peripheral blood, MDA-361 breast cancer cells were labeled with the fluorescent dye CFSE and spiked into 15 mL of normal peripheral blood at a concentration of 1000 cells/mL. Samples were then processed with either a two-compartment separation system based on the differential buoyant density (referred to as 2C separation) or ficoll density gradient centrifugation. The absolute number of cells recovered was determined by manual count using a hemocytometer. Flow cytometry was then performed and the percentage of breast cancer cells in each sample was determined using forward and side scatter characteristics, and fluorescence intensity. The results of a typical experiment are represented in Figure 1. The percentage of breast cancer cells following ficoll density gradient centrifugation was 0.40%, but was 39.42% following 2C separation. Factoring in these percentages and the absolute number of cells recovered per sample, 2C separation resulted in a relative enrichment of 350-fold (Table 1).

Breast cancer cells were spiked into normal peripheral blood as detailed above, and then processed by 2C separation or ficoll density gradient centrifugation. The specimens were then analyzed in a blinded fashion by a skilled cytopathologist and the number of peripheral blood mononuclear cells and breast cancer cells were quantitated. Two-compartment separation routinely reduced the number of peripheral mononuclear cells from a 15mL sample from ?? to less than 500 cells. Recovery of breast cancer cells at spiking concentrations of 10 to 100 cells in 15mL peripheral blood ranged from 30-75%. When samples were processed with ficoll density gradient separation, the large number of peripheral blood cells precluded the reliable identification of recovered breast cancer cells (data not shown).

### Two-compartment separation results in significant reduction in background gene expression in peripheral blood.

Previous studies have shown that low levels of muc 1 (1) and CK19 (2) are expressed in normal PBL. To determine whether 2C separation was able to reduce background expression of

these (or other) cancer-associated genes, samples of normal peripheral blood were processed with either ficoll or 2C separation and analyzed with multi-marker real-time RT-PCR. We observed that when compared to ficoll processed PBL, the Ct values for CK19 and muc1 were reduced by 3 and 10, respectively, when 2C separation was used (Figure 2). The reduction of 10 Ct values for the muc1 gene corresponds to ~1000-fold reduction in gene expression, a value consistent with the 350-fold enrichment observed in the flow cytometry experiments.

Two-compartment separation and multi-marker real-time RT-PCR are capable of detecting breast cancer gene overexpression in the peripheral blood with exquisite sensitivity. In order to determine if enrichment can enhance signals of genes overexpressed in breast cancer cells, cell spiking experiments were performed. A fixed number of MDA-361 cells were spiked into 15mL of normal peripheral blood (1, 10, 150, and 1500 cells/15mL of PBL). Cell spiking was performed using a micromanipulation device to ensure accuracy. Samples were then processed with 2C separation or ficoll density gradient centrifugation and multi-marker real-time RT-PCR. With 2C separation, overexpression of breast cancer-associated genes could be detected when as few as one breast cancer cell was spiked into 15mL of peripheral blood (Figure 3). This corresponds to a sensitivity of one breast cancer cell among  $5 \times 10^8$  peripheral blood cells. Gene overexpression was consistently detected when 10 or more breast cancer cells were spiked into 15mL of peripheral blood.

Table 1. Multi-marker gene panel primer pairs

Gene	Sequence of selected primer pair	Length of amplicon
<b><math>\beta</math>2-microglobin</b>	TGAGTGCTGTCTCC ATGTTTGA	88
<b>PDEF</b>		
<b>Mammaglobin</b>	CGGATGAAACTCT GAGCAATGT	108
<b>CK19</b>	CATGAAAGCTGCCT TGGAAGA	138
<b>Muc1</b>	ACCATCCTATGAGC GAGTACC	107
<b>PIP</b>	GCCAACAAAGCTC AGGACAAC	89
<b>MamB</b>	AGCAGTGTTTCCTC AACCAGTC	126

Table 2 Relative enrichment of breast cancer cells with oncoquick density gradient centrifugation

	<b>Ficoll</b>	<b>Oncoquick</b>
<b>Cell Count</b>	2.2 x 10 <sup>7</sup>	6.4 x 10 <sup>4</sup>
<b>% Breast Cancer Cells</b>	0.4%	39.4%
<b>% Breast Cancer Cell Recovery</b>	59.0%	52.0%
<b>Relative Enrichment</b>	---	350-fold

Figure 1 Selective enrichment of breast cancer cells with Oncoquick

Figure 1A Ficoll

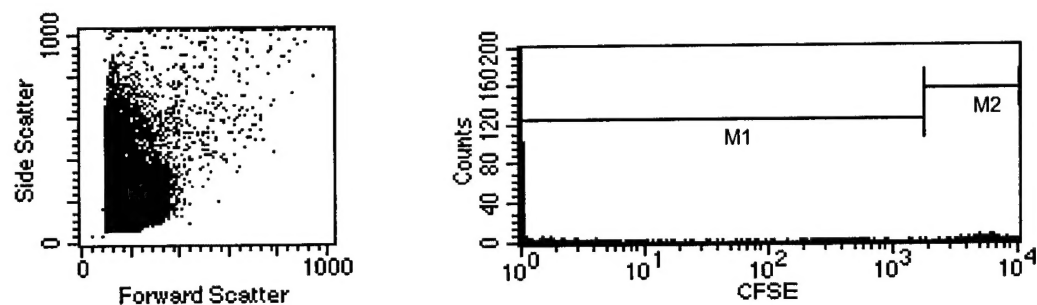


Figure 1B Oncoquick

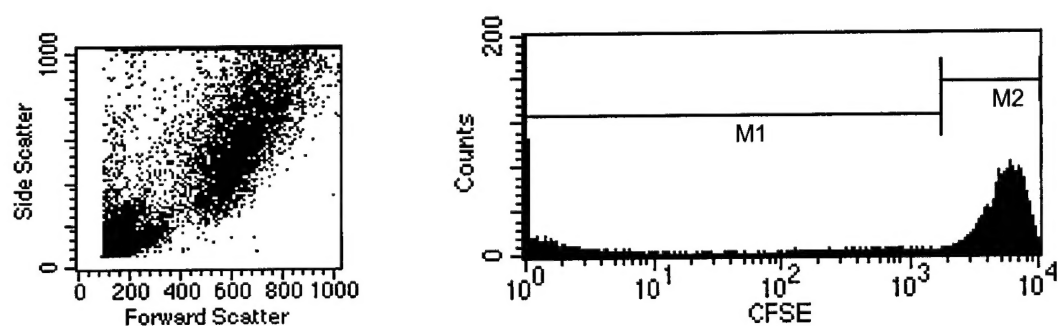


Figure 2 Reduction of molecular background with Oncoquick

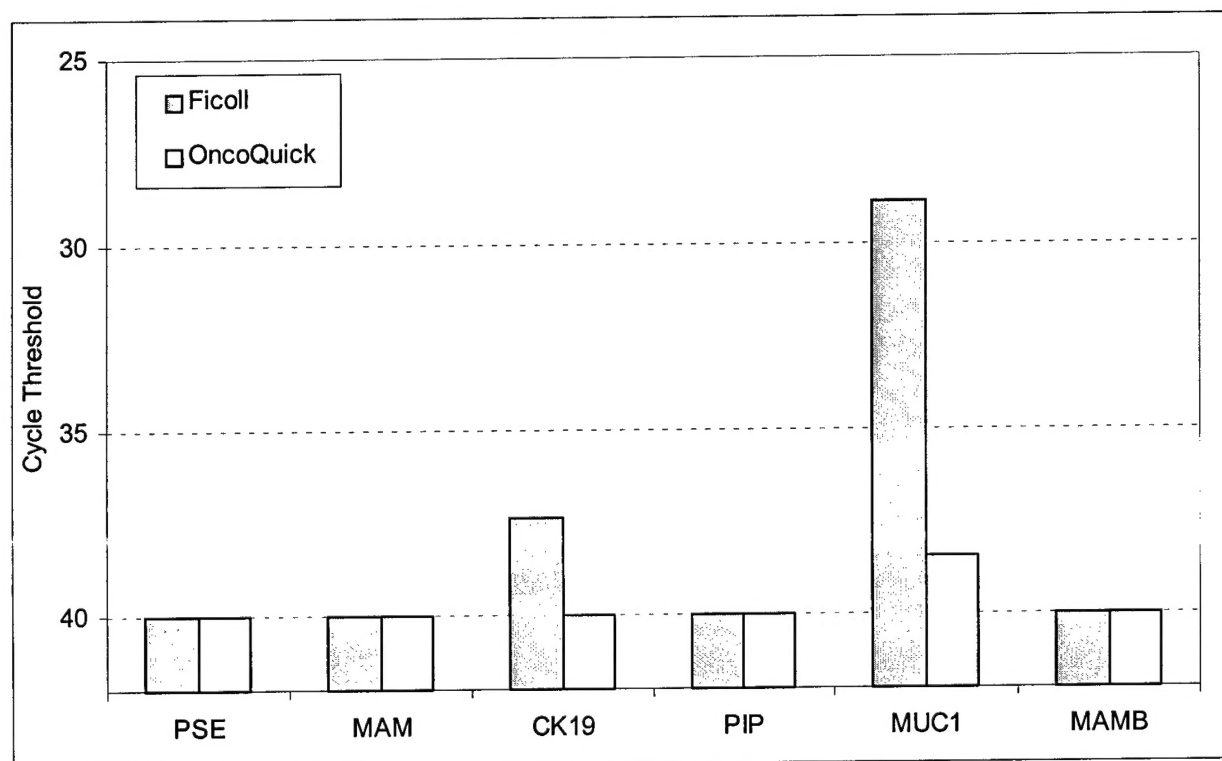
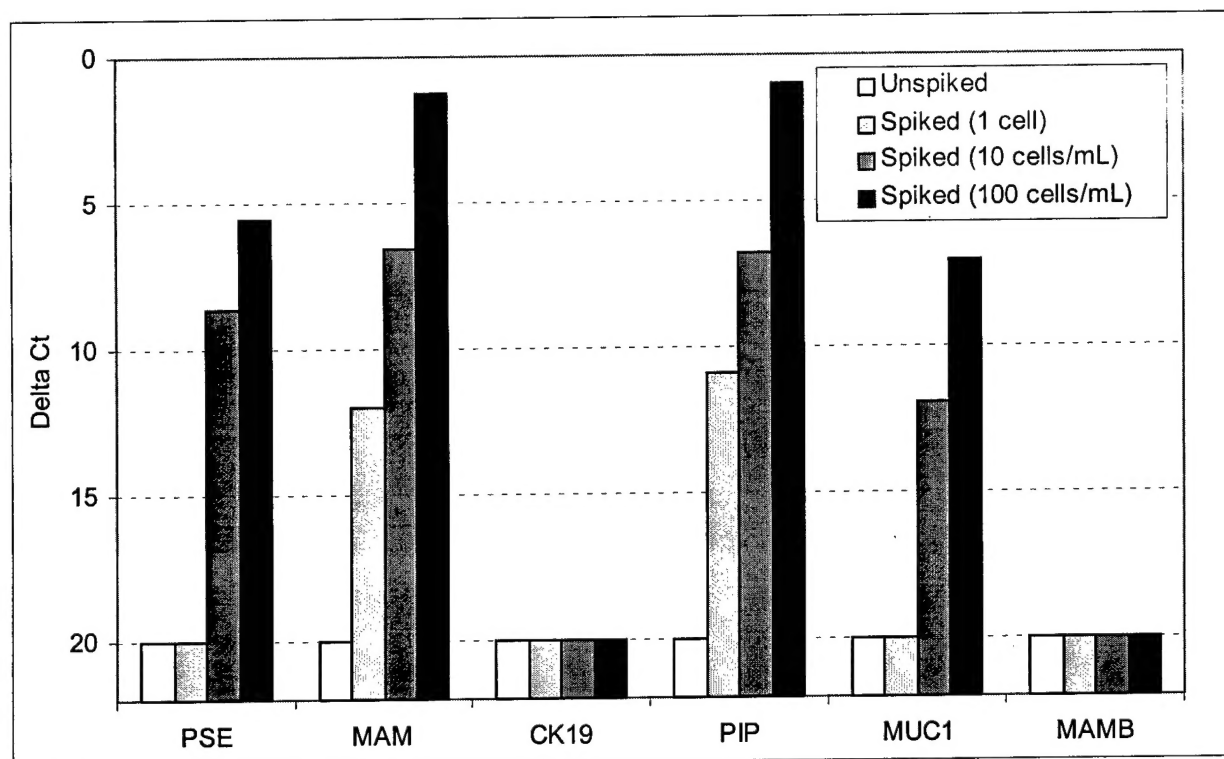




Figure 3 Detection of gene overexpression in peripheral blood samples spiked with breast cancer cells



**Aim 2: Validate the real-time PCR peripheral blood marker assay, and determine whether it can be used to monitor progression or remission of breast cancer.**

The combination of 2-compartment separation and multi-marker real-time RT-PCR can be used for the molecular detection of breast cancer cells in the peripheral blood of Stage IV breast cancer patients.

To assess the ability of 2C separation and multi-marker real-time RT-PCR to detect circulating breast cancer cells in the peripheral blood of breast cancer patients, peripheral blood from twenty healthy volunteers and twenty patients with Stage IV breast cancer was obtained. The samples were enriched for breast cancer cells using 2C separation, and then analyzed by real-time RT-PCR (40 cycles) using a multi-gene marker panel. Gene expression was quantitated by determining cycle threshold values (Figure 4). Sixteen of the twenty Stage IV patients were undergoing systemic chemotherapy at the time of participation. The patient's average age was  $53 \pm 13$  years. Seventy percent of patients ( $n=14$ ) had evidence of gene overexpression. The mean number of positive markers was three. *mam* was the most frequently overexpressed gene (71%), followed by *muc1* (64%), *PIP* (50%), *CK19* (43%), *PDEF* (36%), and *mamb* (29%). Twenty

percent of patients expressed one marker. Of the remaining patients, 5% expressed 2 markers and 45% expressed three or more (Figure 5). Of note, all patients naïve to chemotherapy (n=4) had evidence of gene overexpression in the peripheral blood.

Figure 4 Detection of gene overexpression in Stage IV breast cancer patients

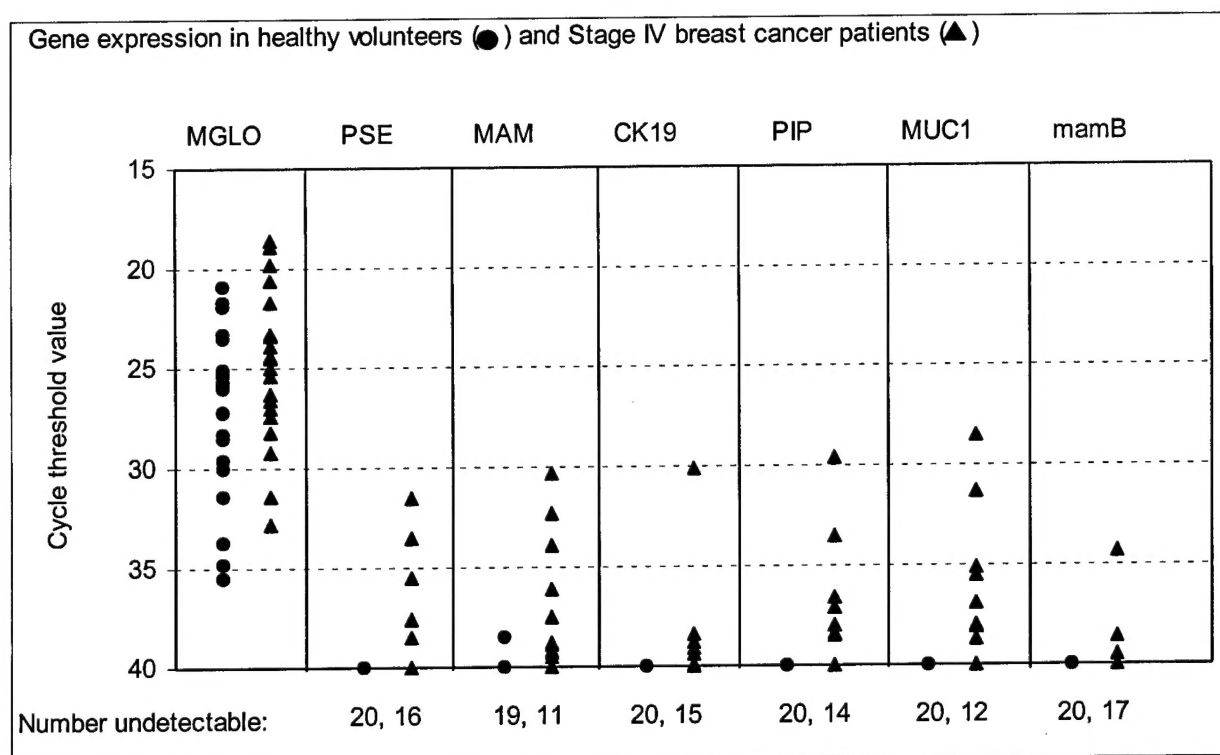
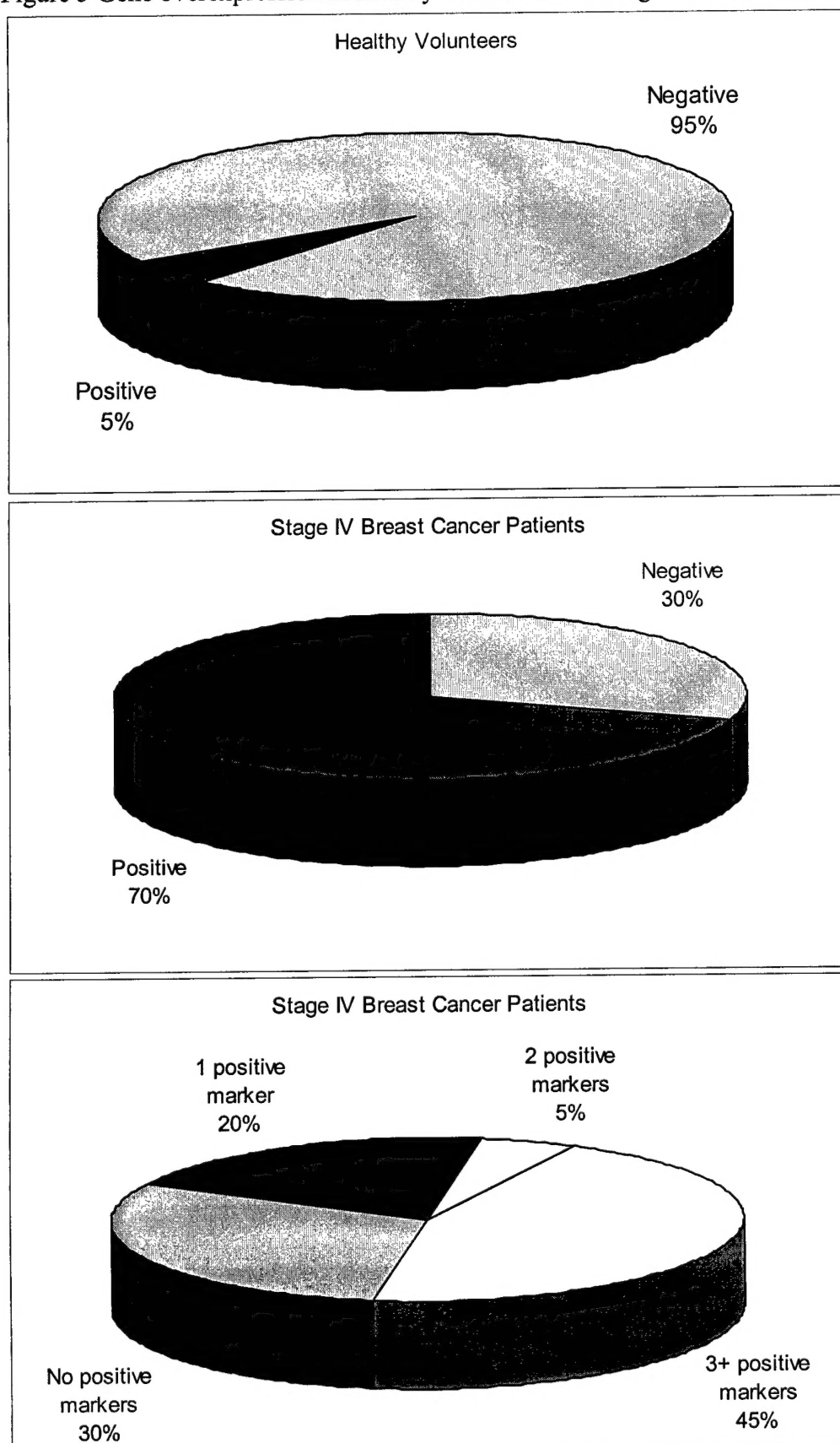


Figure 5 Gene overexpression in healthy volunteers and Stage IV breast cancer patients



## Figure Legends

*Figure 1.* Selective enrichment of breast cancer cells with Oncoquick density gradient centrifugation. MDA-231 cells were labeled with the fluorescent dye CFSE. Labelled cells were then spiked into peripheral blood obtained from healthy volunteers. Samples were then processed with Oncoquick or ficoll density gradient centrifugation. Forward scatter versus side scatter dot plots and CFSE histograms are shown. (A) Samples processed with ficoll density gradient centrifugation. (B) Samples processed with Oncoquick density gradient centrifugation (relative enrichment greater than 350-fold, see Table 2).

*Figure 2.* Reduction of molecular background with Oncoquick density gradient centrifugation. Peripheral blood from healthy volunteers was processed with Oncoquick or ficoll density gradient centrifugation. Multi-marker real-time RT-PCR was performed and gene expression was quantified based on the cycle threshold. With CK19 and MUC1, there is significant expression noted when peripheral blood is processed with ficoll, but this is reduced or absent when blood is processed with Oncoquick. Please note that the y-axis is inverted as there is an inverse relationship between cycle threshold value and gene expression. Also if no gene expression is observed the cycle threshold value is set arbitrarily to 40

*Figure 3.* Detection of gene overexpression in peripheral blood samples spiked with breast cancer cells. MDA-361 breast cancer cells were spiked into 15 mL of peripheral blood using a micromanipulation device at the concentrations indicated. Samples were then processed by Oncoquick density gradient centrifugation and multi-marker real-time RT-PCR. Overexpression of breast cancer-associated genes is observed at the lowest concentration tested, 1 MDA-361 cell in 15 mL peripheral blood.

*Figure 4.* Detection of gene overexpression in Stage IV breast cancer patients. Peripheral blood specimens were obtained from breast cancer patients with known Stage IV disease. The specimens were processed with Oncoquick density gradient centrifugation and multi-marker real-time RT-PCR. Gene expression was quantitated in terms of cycle threshold value. The individual genes are indicated at the top of the figure. Specimen analyses from healthy volunteers are indicated with a circle on the left of each column. Specimen analyses from Stage IV breast cancer patients are indicated with a triangle on the right of each column.

*Figure 5.* Patterns of gene overexpression in healthy volunteers and Stage IV breast cancer patients. The results of the pilot study are summarized in the pie charts. Gene overexpression thresholds were determined as indicated in materials and methods. If gene overexpression is present for any individual marker the specimen is considered to be positive.

### KEY RESEARCH ACCOMPLISHMENTS

- Identification of a reliable method (two-compartment gradient separation) for molecular detection of tumor cells in the peripheral blood of breast cancer patients.
- Detection of tumor cells in peripheral blood of breast cancer patients.

### REPORTABLE OUTCOMES

Manuscript in preparation:

Molecular detection of breast cancer cells in the peripheral blood of advanced stage breast cancer patients using multi-marker real-time RT-PCR and a novel density-gradient separation technology

### CONCLUSIONS

In our pilot study, 70% of Stage IV breast cancer patients and 100% of those naïve to treatment had evidence of breast cancer-associated gene overexpression. We suspect that the statistical significance and clinical relevance of these correlative findings would be substantiated in a larger patient cohort. Given the encouraging results described in this paper, it is easy to imagine that the combined technologies could be used to monitor patients' response to treatment. Ultimately, further refinement is likely to allow for breast cancer screening and staging of patients with less advanced disease.

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